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Effects of elevated CO_2 on the extractable amino acids of leaf litter and fine roots

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Summary

- Elevated atmospheric CO_2 concentrations can change chemistry and input rate of plant tissue to soil, potentially influencing above- and below-ground biogeochemical cycles. Given the important role played by leaf and root litter chemistry in controlling ecosystem function and vulnerability to environmental stresses, we investigated the hydrolyzable amino acid distribution and concentration in leaf and fine root litter among control and elevated CO_2 treatments at the Rhinelander free air CO_2 enrichment (FACE) experiment (WI, USA).
- We extracted hydrolyzable amino acids from leaf litter and fine (< 2 mm) roots at three depths for both control and elevated CO_2 plots.
- We found that elevated CO_2 decreased the proportion of total leaf amino acid carbon (C), but had no effect on total leaf amino acid nitrogen (N). There was no treatment effect for total root amino acid N or amino acid C for any depth.
- The decrease in leaf amino acids is probably a result of the shift of protein compounds to more structural compounds. Despite the decrease in leaf amino acid C concentrations, the overall increase in annual plant production under elevated CO_2 would result in an increase in plant amino acids to the soil.

Introduction

Rising atmospheric CO_2 concentrations have the potential to change both the chemistry and input rate of plant and microbial organic matter to soil, causing fundamental changes in both above- and below-ground biogeochemical cycles (Holmes *et al.*, 2003; Norby *et al.*, 2005; Kubiske *et al.*, 2007; Talhelm *et al.*, 2009). An initial response of forests to elevated CO_2 is an increase in net primary productivity (NPP) for both leaves and fine roots (Norby *et al.*, 2005; Pregitzer *et al.*, 2008). The increase in CO_2 influences carbon (C) apportionment into plant tissue and results in the preferential allocation of the added C leaf and fine root tissue (Norby *et al.*, 2002). The change in carbon allocation also influences key precursors for the production of plant metabolites and may, therefore, cause changes in the fluxes of those metabolites within the plant, as well as those that are released into the soils (King *et al.*, 2001; Lindroth, 2010). Understanding how the concentrations of individual classes of organic compounds are altered with environmental changes, such as elevated CO_2, is critical for predicting the amounts of these components in future global change scenarios and their coupled impact on soil organic matter (SOM) dynamics (Ehleringer *et al.*, 2000).

Because elevated CO_2 results in changes in NPP to both leaves and fine roots, fine root biochemistry is also likely to undergo changes; however, this has been studied in less detail. The influence of elevated CO_2 on plant tissue chemistry has been the subject of many studies and results have been variable: decreases in foliar nitrogen (N) (McGuire *et al.*, 1995; Liu *et al.*, 2005; Couture *et al.*, 2012), increases in carbohydrate concentrations (Lincoln *et al.*, 1993; Peñuelas & Estiarte, 1998; Lindroth *et al.*, 2001), increases as well as invariance in condensed tannin and lignin concentrations (Coûteaux *et al.*, 1999; Finzi *et al.*, 2001; Parsons *et al.*, 2008; Liu *et al.*, 2009; Couture *et al.*, 2012), and altered plant starch and fiber concentrations (Lindroth *et al.*, 2001; Meehan *et al.*, 2010). These responses to elevated CO_2, however, have a strong dependence on the clonal variety of a species (Parsons *et al.*, 2008).

Fine root biochemistry and decomposition processes are essential to the overall organic matter budgets of forest ecosystems (McClaugherty *et al.*, 1984; Jackson *et al.*, 1997; Rasse, 2002). Analyses of a number of forest free air CO_2 enrichment (FACE) experiments demonstrate that increased C entering the below-ground system (e.g. increased root biomass) under elevated CO_2 results in greater N uptake, including in N-limited ecosystems (Finzi *et al.*, 2007; Zak *et al.*, 2007; Norby & Zak, 2011). At the sweetgum (*Liquidambar styraciflua*) forest FACE experiment at Oak Ridge National Laboratory, Norby & Iversen (2006) found that an increase in production of fast-turnover fine roots maintained the N uptake. Therefore, any changes to the production of or turnover rate of fine root tissue could feed back to the ability of forest systems under elevated CO_2 to respond to N limitation (Norby & Iversen, 2006; Iversen & Norby, 2008).

Protein amino acids make up *c.* 10% of the total dry mass of plant roots and shoots (Rejsek *et al.*, 2010) and their input to soil via root turnover and leaf litter fall represents the biggest single N input into most terrestrial ecosystems (Jones *et al.*, 2005). Additionally, the largest source of free amino acids in the soil is from above-ground litter and dead roots (Jones & Kielland, 2002), and they provide a direct source of N for plants and microbes. Johnson & Pregitzer (2007) examined the free amino acids in the soil at the Rhinelander FACE site and found no significant responses of the free amino acids to the elevated CO_2 treatment, although a few individual amino acids showed significant changes with elevated CO_2, potentially suggesting that the amino acids in the plant litter are not affected by elevated concentrations of CO_2. Larson *et al.* (2002) also found that there was no significant change in leucine aminopeptidase, an enzyme used in protein degradation, with elevated CO_2 at the Rhinelander FACE site, further suggesting that protein and amino acid input from dead root and leaf litter were unchanged with elevated CO_2.

Given the important role played by leaf and root litter chemistry in the cycling of C and N in forest ecosystems, the variable responses of litter chemistry to elevated CO_2 reported to date, and the need for further detailed chemical studies addressing plant amino acid N and C under elevated CO_2 conditions, we investigated hydrolyzable amino acids in leaf and fine root litter among control and elevated CO_2 treatments at the Rhinelander FACE (WI, USA) experiment. Specifically, we sought to evaluate whether the elevated CO_2 treatment had an effect on the type and abundance of amino acids in the leaf and root litter. Given previously observed redistribution of C and N in leaf biomolecules resulting from elevated CO_2, (Lindroth *et al.*, 2001; Oksanen *et al.*, 2001; Holton *et al.*, 2003; Liu *et al.*, 2005; Mattson *et al.*, 2005) we expected that elevated CO_2 would result in lower amino acid concentrations g^{-1} C but not g^{-1} N of leaf and root litter.

Materials and Methods

Site description

The study was conducted at the Rhinelander Free-Air CO_2 Enrichment (Rhinelander FACE) site located in Rhinelander (WI, USA). Soils at this site are sandy loam and described as a mixed, frigid, coarse loamy Alfic Haplorthod (Padus Series) (Dickson *et al.*, 2000). Exact details regarding the design and performance of the experiment can be found in Dickson *et al.* (2000). Briefly, 12 FACE rings (30 m diameter) spaced 100 m apart on a 32 ha field were established and divided into east and west halves. The sampling for this study was in the east half of the rings, which were planted with five genotypes of aspen (*Populus tremuloides* Michx.) that range in O_3 sensitivity and CO_2 responsiveness (highly tolerant to highly sensitive); the western half of the rings were divided in half and planted with aspen and sugar maple and aspen and birch, although sampling was not done in those sections. Treatment started in 1997 with 1-yr-old plants and continued until summer 2009 when the trees were harvested and a regeneration experiment began. The fumigation experiment combines CO_2 and O_3 exposures to each ring, resulting in three control rings, three CO_2 rings (target concentration of 560 ppm), three O_3 rings (target concentration of 55 ppb), and three combined CO_2 and O_3 rings (Dickson *et al.*, 2000; Karnosky *et al.*, 2005). Sampling for this study was performed in July 2009 as one of the last set of samples collected before tree harvest, close to the time when the experiment was shut down.

Sampling

Samples were collected from the outer edge of the eastern half of each ring, which was composed of five aspen genotypes. Five field replicate samples were taken in each of the three control and elevated CO_2 treatment rings. Soil cores to a depth of 25 cm were taken with a 5-cm-diameter barrel soil corer (AMS, American Falls, ID, USA) and divided into 0–5, 5–15 and 15–25 cm sections in the field and stored over ice for transport to the laboratory for processing. In the laboratory, soils were sieved in field-moist condition (4 mm), and living and dead roots were hand-picked from each depth interval as a combined sample, washed, separated into < 2 mm and > 2 mm (fine and coarse roots; coarse roots not included in present study) diameter sizes, air-dried and ground with a stainless steel mill (Retcsh, Haan, Germany).

Leaf litter samples were collected off the ground from five 0.04 m^2 quadrats immediately adjacent to the soil cores and dried at 50°C for 48 h on site. It is important to note that the leaf litter sample represents partially decomposed leaf litter, as it is litter from the preceding fall and would have undergone some decomposition over the winter and spring. Nonleaf debris was removed from litter, and leaf samples were pooled and ground before amino acid and elemental analysis. Dry weight percentage C and N values were determined for leaf and root litter using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a Sercon 20-20 isotope ratio mass spectrometer (Sercon Ltd, Cheshire, UK).

Amino acid extraction and analysis

Total (bound and water-soluble) amino acids were extracted from leaf litter and roots according to a modified method by Amelung & Zhang (2001). Briefly, samples amounting to 2–4 mg organic carbon were hydrolyzed with 5 ml of 6 M HCl for 12 h at 105°C. After hydrolysis, the mixture was spiked with α-aminobutyric acid as a standard. The hydrolysates were filtered (pore size 1.6 μm) and evaporated to dryness. Amino acids were redissolved in 0.05 M HCl and trapped on an elution column packed with Dowex 50 W X8 cation exchange resin (100–200 mesh size) (Sigma Aldrich), which allowed for removal of dissolved metals by rinsing with oxalic acid. Purified amino acids were eluted with NH_4OH into flasks and then dried under a gentle flow of purified air.

Derivatization of the amino acids, to permit GC analysis, was performed according to Macko *et al.* (1997). Briefly, a subsample of the amino acid solution was placed in an ashed and solvent-rinsed screw-cap vial and evaporated to dryness under N_2. Samples were esterified with acidified isopropanol and then acetylated

by the addition of trifluoroacetic anhydride. With this hydrolysis and derivatization procedure, it was not possible to measure arginine, methionine, histidine, cysteine, and tryptophan. Asparagine and glutamine decompose during hydrolysis and contribute to aspartic and glutamic acids, respectively. Amino acid concentrations were presented individually and in terms of groupings, where nonpolar amino acids included alanine, glycine, valine, leucine, isoleucine, proline, and phenylalanine; polar amino acids included threonine, serine, and tyrosine; acidic acids included aspartic acid and glutamic acid; and basic amino acids included only lysine.

Samples were analyzed using a Shimadzu GC17A gas chromatograph interfaced to a Shimadzu QP5050A quadrupole mass spectrometer scanned over the range m/z 35–550. An 18-amino-acid standard suite (Sigma Aldrich) was used for GC calibration using selected mass extraction curves for each amino acid quantified. The capillary column used was a 30 m RTX-5MS column (Restek, Bellefonte, PA, USA) and the GC oven temperature was programmed from 60 to 280°C at 10.5°C min^{-1} and held for 7 min.

Statistical analyses

Leaf litter and root data from individual quadrats were pooled within each ring for analytical analysis (thus replicated at the ring level). The data set consisted of ring replicates of each sample of leaf litter and root amino acids and C and N data from the two different atmospheric treatments: ambient and elevated CO_2 ($n = 3$ for both control ambient and elevated CO_2). Amino acid data are presented as normalized to plant N and C (e.g. g amino acid C g^{-1} root C); this indicated the percentage of the total root C that is made up of amino acid C. The experimental design of the FACE experiment is a randomized complete block design with two or three factorial treatments similar to that of Talhelm *et al.* (2009) and King *et al.* (2001). One factor, CO_2, was used for the leaf treatment and a second factor, depth, was added for the root data. Data were analyzed with a fixed-effects one-way ANOVA model where type III sums of squares were used within the GLM procedure in the least-squares means adjustment for multiple comparisons: Tukey–Kramer was used to compare treatments and depths. Analysis was done with SAS statistical software (SAS 9.2, SAS Institute Inc., Cary, NC, USA). An $\alpha = 0.05$ value was used to determine statistical significance.

Results

C and N in leaf and root litter

Elevated CO_2 did not change the % C or % N in the leaf litter. Mean values for control and elevated CO_2 were 46.6 and 46.7%, respectively for C and 1.5 and 1.3% for N (Table 1). There was a significant effect on the mean C : N ratios, increasing under elevated CO_2 (Table 1).

Elevated CO_2 significantly increased % C above control values only in the 0–5 cm depth fine roots, living plus dead, raising it from 25.8 to 34.1% (Table 1). Elevated CO_2, however, did not have an effect on the % N in the fine root tissue at any depth (Table 1). Mean values of elevated CO_2 fine root % C were lower than the control % C in the lower 5–25 cm fine root depths; however, the difference was not significant. The lower % C also resulted in lower C : N ratios for the elevated CO_2 treatment at the lower root depths, but this difference was only significant for the lowest 15–25 cm depth (Table 1). Additionally, the % N in roots decreased with depth in both treatments, while % C increased for the control but varied nonsystematically for the elevated CO_2 treatment. The C : N ratio increased with depth of roots in both the control and elevated CO_2 treatments (Table 1).

Table 1 The % carbon (C) DW, % nitrogen (N) DW, and elemental C : N ratio (± SE) for leaf litter and roots collected from the aspen (*Populus tremuloides*) section of each ring at the free air CO_2 enrichment (FACE) experiment in Rhinelander, WI, USA

	% C	% N	C : N
Leaf litter			
Control	46.7 ± 0.5	1.5 ± 0.08	31.3 ± 1.5^{a}
CO_2	46.8 ± 0.7	1.3 ± 0.05	37.3 ± 2.2^{b}
Fine roots (0–5 cm)			
Control	$25.8 \pm 0.3^{a,*}$	1.1 ± 0.02*	24.5 ± 0.5*
CO_2	$34.1 \pm 0.6^{b,*}$	1.1 ± 0.05*	30.5 ± 1.2*
Fine roots (5–15 cm)			
Control	$36.5 \pm 0.1^{\delta}$	$0.9 \pm 0.04^{\delta}$	$43.0 \pm 2.6^{\delta}$
CO_2	$30.8 \pm 0.3^{\delta}$	$0.9 \pm 0.05^{\delta}$	34.8 ± 2.1*
Fine roots (15–25 cm)			
Control	$38.7 \pm 1.2^{\delta}$	$0.7 \pm 0.01^{\infty}$	$58.3 \pm 2.1^{a,\infty}$
CO_2	33.8 ± 0.3*	$0.7 \pm 0.07^{\infty}$	$45.8 \pm 3.9^{b,\delta}$

Letters indicate significant ($\alpha = 0.05$) treatment effects
Symbols indicate significant ($\alpha = 0.05$) depth effects within a treatment.

Leaf litter and root amino acids

Elevated CO_2 resulted in a significant overall reduction in the proportion of leaf litter C extractable as amino acids (mg amino acid C g^{-1} leaf litter C) for most individual amino acids (Table 2). The extracted amino acid C represented $6.0 \pm 0.3\%$ of the overall leaf litter C for the control and only $4.8 \pm 0.2\%$ for the elevated CO_2 treatment. The dominant amino acids contributing to leaf C for both the control and elevated CO_2 treatments were leucine and glutamic acid (glutamic acid + glutamine).

Elevated CO_2 had no impact on the total leaf litter amino acid N extracted (Table 2), although a few amino acids exhibited significant differences. Specifically, alanine N and valine N showed a significant proportional increase with CO_2 (Table 2). The extracted amino acid N represented 48.6 and 47.3% of the overall leaf litter N for the control and elevated CO_2 treatments, respectively. Dominant amino acids contributing to the overall N for the control treatment were glutamic acid (glutamic acid + glutamine), aspartic acid (aspartic acid + asparagine), and glycine. For the elevated CO_2 treatment, the dominant amino acids contributing to the overall leaf litter N were glycine, alanine, and glutamic acid (glutamic acid + glutamine).

Table 2 Normalized aspen (*Populus tremuloides*) leaf litter amino acid carbon (C) and nitrogen (N) (± SE) for the treatments at the Rhinelander free air CO_2 enrichment (FACE) experiment

	Normalized leaf litter amino acid N (mg g^{-1} leaf N)		Normalized leaf litter amino acid C (mg g^{-1} leaf C)	
Amino acid groups	Control	Elevated CO_2	Control	Elevated CO_2
Nonpolar	**249.3 ± 2.5**	**253.7 ± 3.9**	**32.4 ± 1.5**	**26.9 ± 1.1***
Alanine	45.8 ± 0.5	49.7 ± 0.4*	3.9 ± 0.1	3.5 ± 0.1
Glycine	52.0 ± 0.9	53.8 ± 0.6	2.9 ± 0.1	2.5 ± 0.1*
Valine	31.6 ± 0.6	34.2 ± 0.4*	4.4 ± 0.2	4.0 ± 0.2
Leucine	44.5 ± 0.2	43.2 ± 0.9	7.5 ± 0.4	6.0 ± 0.2*
Isoleucine	22.2 ± 0.2	22.6 ± 0.4	3.7 ± 0.1	3.2 ± 0.1*
Proline	31.9 ± 0.9	29.9 ± 1.1	4.5 ± 0.2	3.5 ± 0.2*
Phenylalanine	21.5 ± 0.5	20.2 ± 0.4	5.4 ± 0.4	4.2 ± 0.1
Polar	**86.4 ± 1.8**	**87.6 ± 0.6**	**9.8 ± 0.4**	**8.1 ± 0.3***
Threonine	33.2 ± 0.5	33.8 ± 0.6	3.7 ± 0.1	3.2 ± 0.2*
Serine	43.7 ± 0.9	45.2 ± 0.6	3.7 ± 0.1	3.2 ± 0.1*
Tyrosine	9.5 ± 0.8	8.7 ± 0.3	2.4 ± 0.2	1.8 ± 0.1
Acidic	**107.3 ± 1.9**	**96.7 ± 4.0**	**13.6 ± 0.4**	**10.2 ± 0.6***
Aspartic acid	52.1 ± 1.0	47.5 ± 2.2	5.9 ± 0.3	4.4 ± 0.3*
Glutamic acid	55.2 ± 1.8	49.2 ± 1.7	7.7 ± 0.2	5.7 ± 0.4*
Basic	**43.8 ± 2.4**	**35.3 ± 2.6**	**3.7 ± 0.3**	**2.5 ± 0.2***
Lysine	43.8 ± 2.4	35.3 ± 2.6	3.7 ± 0.3	2.5 ± 0.2*
Total	**486.8 ± 7.0**	**473.3 ± 11.2**	**59.6 ± 2.5**	**47.7 ± 2.3***

*Significant differences ($\alpha = 0.05$) between control and elevated CO_2.

Elevated CO_2 did not have an effect on the total extractable amino acid C in root litter (mg amino acid C g^{-1} root litter C). Although not statistically significant (at $P < 0.05$), differences existed for a few amino acids at the 0–5 cm depth (Table 3). Specifically, valine C and tyrosine C both decreased with elevated CO_2 ($P > 0.1$), while alanine C also showed a decrease ($P = 0.11$). There was an overall decrease in root amino acid C at the 0–5 cm depth ($P = 0.19$), but that trend was not consistent with depth. In the lower depths, there was a slight increase in the average root amino acid C; however, those differences were not significant either (Table 3). The dominant amino acids that contributed to the overall root % C were glutamic acid (glutamic acid + glutamine), aspartic acid (aspartic acid + asparagine), and leucine. This was consistent across depth and treatment.

The total extractable amino acid C represented 6.3 ± 0.5% of the overall root C at the 0–5 cm depth for the control treatment and 5.3 ± 0.3% for the elevated CO_2 treatment. At the 5–15 cm depth, extractable amino acid C represented 3.7 ± 0.07 and 4.0 ± 0.5% for the control and CO_2 treatments, respectively. At the 15–25 cm depth, the amino acid C contributions to the overall root C were 2.7 ± 0.3 and 3.1 ± 0.3%, for the control and elevated CO_2 treatments, respectively. For the control treatment, amino acid C decreased significantly with depth between the 0–5 cm and 5–15 cm depths; however, there was no difference between the 5–15 and 15–25 cm depths (Fig. 1a). This trend was consistent for all individual root amino acid C for the control treatment. There was a general decrease in amino acid C with depth also with elevated CO_2, but the decrease was significant between the 0–5 and 15–25 cm depths in root amino acid C, and the 5–15 cm depth was an intermediary amount between the two depths. The trend existed for all individual amino acid N amounts, except for tyrosine for the elevated CO_2 treatment (Table 3).

Elevated CO_2 had no effect on amino acid N (Table 4); nor did depth have a significant effect on amino acid N (Fig. 1b). The dominant amino acids contributing to the overall root % N were glutamic acid (glutamic acid + glutamine), aspartic acid (aspartic acid + asparagine), glycine and alanine (Table 4). This was consistent for control and CO_2 treatments, as well as for depth. Amino acid N represented between 40.5 ± 1.2 and 46.1 ± 4.3% for both treatments and across all depths (Table 4).

Discussion

Elevated CO_2 shifts leaf litter to lower quality substrate

In our measurement of accumulated leaf litter, we did not observe the decrease in foliar N content with elevated CO_2 that was reported in other studies from the Rhinelander FACE experiment (Liu *et al.*, 2005; Couture *et al.*, 2012). However, the increase in the C : N ratio observed in aspen leaf litter (Table 1) under elevated CO_2 was consistent with previous results from this same site (Lindroth *et al.*, 2001; Chapman *et al.*, 2005; Liu *et al.*, 2005). In our study, the increase in C : N was primarily caused by a decrease in foliar N. However, overall leaf litter C : N values observed in this study were slightly lower than previously published results from Rhinelander FACE leaf analyses. The reason for this may be that in the present study we analyzed forest floor litter, rather than recent litter fall or leaves sampled from trees, which potentially contained the accumulated effects of a few seasons of decay, resulting in microbial N accumulation and C loss (Köchy & Wilson, 1997).

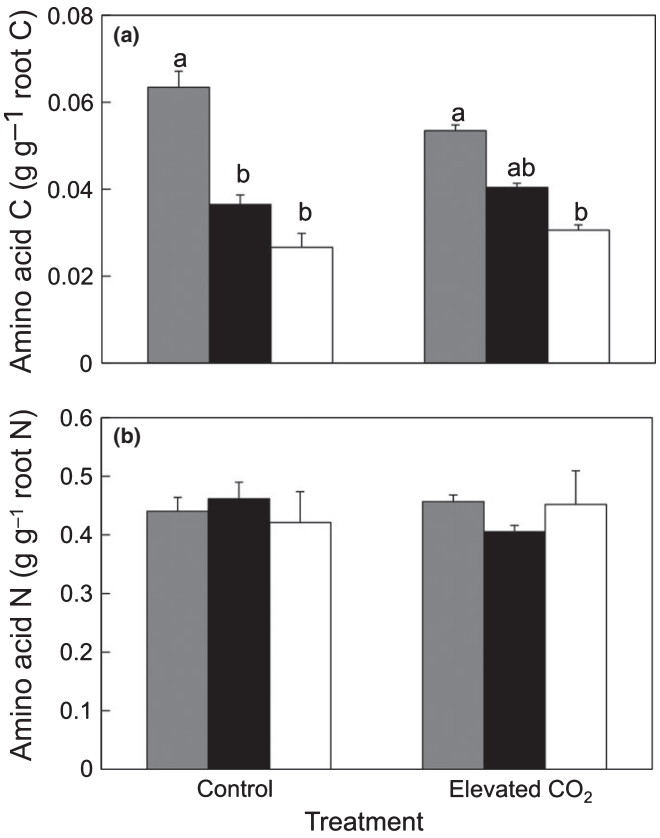

Fig. 1 Normalized aspen (*Populus tremuloides*) root amino acid carbon (C) (a) and root amino acid nitrogen (N) (b) across depths among the treatments at the Rhinelander free air CO_2 enrichment (FACE) experiment. Gray bars, 0–5 cm depth; black bars, 5–15 cm depth; white bars, 15–25 cm depth. Letters indicate significant differences across depth within each treatment; error bars, ± 1 SE. No statistical significance was found for the root litter amino acid N.

Consistent with our initial hypothesis, elevated CO_2 resulted in a lower proportion of leaf litter C as amino acid C with respect to control. Decreases in amino acid concentrations as a result of elevated CO_2 have also been observed in a variety of plants, including cactus (Wang & Nobel, 1996), cotton (Sun *et al.*, 2009), spring wheat (Högy *et al.*, 2010), and barley (Sicher, 2008). These results are in keeping with findings demonstrating a relative shift toward accumulation of structural compounds with overall poorer nutrient quality in leaf tissue grown under CO_2 from this FACE site (Lindroth *et al.*, 2001; Oksanen *et al.*, 2001; Holton *et al.*, 2003; Karnosky, 2003; Liu *et al.*, 2005). The accumulation of structural compounds, however, is not always a consistent response to growth under elevated CO_2. For example, at the Rhinelander FACE site, Mattson *et al.* (2005) found that aspen leaves under elevated CO_2 were enriched in phenolic glycosides, but not significantly in other aromatic products such as tannins, lignin, and carotenoids. Parsons *et al.* (2008), on the other hand, found aspen leaves from the Rhinelander FACE site enriched in soluble and bound tannins. Wustman *et al.* (2001) demonstrated that some aspen clones under elevated CO_2 at the Rhinelander FACE site actually suppressed the flow of carbon through the phenylpropanoid pathway, which uses phenylalanine and tyrosine as precursors to cinnamic acids. In the present study, elevated CO_2 had a slight influence on the overall proportion of leaf litter N as amino acid N (Table 2), which indicates that only the flow of C in the leaf is being repartitioned and not the flow of N. Additionally, as the dominant amino acids were also not altered under elevated CO_2, it is most likely that the amino acid biochemical pathways were not being altered either.

Because these leaf litter samples have undergone several months of decay, some of the difference in amino acid concentration between the control and the elevated CO_2 treatments may be the result of potential differences in the decomposition patterns under the fumigation treatments. However, this scenario is unlikely, as previous litter bag decomposition studies at Rhinelander FACE indicated no treatment effect on rate of decomposition for roots (Chapman *et al.*, 2005) or leaves (Liu *et al.*, 2007) isolated from this site. Contradicting these results, Parsons *et al.* (2008) found that decomposition was slower for aspen leaves in the elevated CO_2 treatment than in the control treatment (Parsons *et al.*, 2008). A slower decomposition would indicate that the initial amino acid differences were larger or that there was selective protein decomposition under elevated CO_2.

Elevated CO_2 has little impact on the root amino acids

In the present study we found that elevated CO_2 had little impact on extractable root amino acid C or amino acid N, other than for a few individual amino acids in the 0–5 cm depth. Similarly, King *et al.* (2005) found little effect of elevated CO_2 on fine root C metabolite chemistry in aspen trees at the Rhinelander FACE site. The lack of change in amino acid C or N with the aspen roots in the current study may result from the fact that the samples were collected after the trees had undergone elevated CO_2 treatments for more than a decade, resulting in a more stable response, as opposed to the more dynamic responses of trees at a younger growth stage (Bazzaz *et al.*, 1990). Lack of treatment effect may also be the result of sampling date. New root production can peak at midsummer at the same latitude as the Rhinelander FACE site (Hendrick & Pregitzer, 1992a) and this corresponded with the trends observed with soluble sugar concentrations in the soil as well as root activity and growth in Johnson & Pregitzer (2007). It is possible that significant differences might exist earlier or later in the season for root amino acids.

Although elevated CO_2 did not have an impact on individual root amino acid concentrations, depth had a significant effect on amino acid C, but not amino acid N. There was an interesting difference between elevated CO_2 and control treatments in the change from 0–5 and 5–15 cm depths. For the control, the drop in concentration was large and significant between the 0–5 and 5–15 cm depths, but not so for the elevated CO_2 treatments, which resulted in only a small difference in proportion of amino acids with depth. The overall decrease in amino acids with depth may also be a combined result of the proportion of living and dead fine roots in the < 2 mm size class. Additionally, roots in deeper soils (10–20 cm) also tend to be thicker and have higher tissue density (Hendrick & Pregitzer, 1992b; Fahey & Hughes, 1994). This characteristic may be related to different functions of roots at different soil depths, and different function could

Table 3 Normalized aspen (*Populus tremuloides*) root amino acid carbon (C) (± SE) across treatments at the Rhinelander free air CO_2 enrichment (FACE) experiment within the different groups of amino acids for each root depth: 0–5 cm, 5–15 cm and 15–25 cm

	Normalized root amino acid C (mg g^{-1} root C)					
	0–5 cm		5–15 cm		15–25 cm	
Amino acid groups	Control	Elevated CO_2	Control	Elevated CO_2	Control	Elevated CO_2
Nonpolar	**34.0 ± 2.6^{a}**	**29.4 ± 1.4^{a}**	**20.0 ± 0.4^{b}**	**22.2 ± 2.9^{a,b}**	**15.6 ± 2.1^{b}**	**17.1 ± 1.5^{b}**
Alanine	4.6 ± 0.3^{a}	3.9 ± 0.2^{a}	2.6 ± 0.1^{b}	3.0 ± 0.3^{a,b}	1.9 ± 0.3^{b}	2.2 ± 0.2^{b}
Glycine	3.3 ± 0.2^{a}	2.9 ± 0.2^{a}	1.9 ± 0.1^{b}	2.1 ± 0.3^{a,b}	1.3 ± 0.2^{b}	1.5 ± 0.2^{b}
Valine	5.1 ± 0.3^{a}	4.3 ± 0.1^{a}	3.0 ± 0.1^{b}	3.3 ± 0.5^{a,b}	2.3 ± 0.3^{b}	2.6 ± 0.3^{b}
Leucine	7.1 ± 0.6^{a}	6.4 ± 0.2^{a}	4.5 ± 0.1^{b}	4.8 ± 0.7^{a,b}	3.4 ± 0.4^{b}	3.7 ± 0.4^{b}
Isoleucine	3.7 ± 0.3^{a}	3.2 ± 0.2^{a}	2.2 ± 0.1^{b}	2.4 ± 0.4^{a,b}	1.7 ± 0.3^{b}	1.9 ± 0.2^{b}
Proline	5.1 ± 0.4^{a}	4.3 ± 0.3^{a}	3.0 ± 0.1^{b}	3.3 ± 0.4^{a,b}	2.5 ± 0.3^{b}	2.5 ± 0.2^{b}
Phenylalanine	5.1 ± 0.5^{a}	4.5 ± 0.4^{a}	2.9 ± 0.1^{b}	3.3 ± 0.4^{a,b}	2.3 ± 0.4^{b}	2.7 ± 0.2^{b}
Polar	**10.2 ± 0.9^{a}**	**8.4 ± 0.8^{a}**	**5.7 ± 0.1^{b}**	**6.5 ± 0.6^{a,b}**	**4.1 ± 0.7^{b}**	**5.0 ± 0.6^{b}**
Threonine	4.4 ± 0.3^{a}	3.8 ± 0.4^{a}	2.4 ± 0.1^{b}	2.9 ± 0.2^{a,b}	1.6 ± 0.3^{b}	2.2 ± 0.3^{b}
Serine	4.0 ± 0.3^{a}	3.5 ± 0.3^{a}	2.5 ± 0.1^{b}	2.7 ± 0.3^{a,b}	1.7 ± 0.3^{b}	2.1 ± 0.3^{b}
Tyrosine	1.9 ± 0.2^{a}	1.2 ± 0.2^{a}	0.8 ± 0.1^{b}	0.8 ± 0.1^{a,b}	0.8 ± 0.1^{b}	0.7 ± 0.1^{b}
Acidic	**16.1 ± 1.5^{a}**	**13.2 ± 0.7^{a}**	**9.1 ± 0.1^{b}**	**9.9 ± 1.4^{a,b}**	**5.7 ± 0.9^{b}**	**7.2 ± 1.1^{b}**
Aspartic acid	7.5 ± 0.6^{a}	6.2 ± 0.3^{a}	4.4 ± 0.1^{b}	4.7 ± 0.7^{a,b}	2.7 ± 0.4^{b}	3.5 ± 0.6^{b}
Glutamic acid	8.6 ± 0.8^{a}	7.0 ± 0.4^{a}	4.8 ± 0.1^{b}	5.2 ± 0.7^{a,b}	3.0 ± 0.4^{b}	3.7 ± 0.6^{b}
Basic	**3.1 ± 0.3^{a}**	**2.4 ± 0.4^{a}**	**1.7 ± 0.1^{b}**	**1.8 ± 0.4^{a,b}**	**1.3 ± 0.1^{b}**	**1.2 ± 0.1^{b}**
Lysine	3.1 ± 0.3^{a}	2.4 ± 0.4^{a}	1.7 ± 0.1^{b}	1.8 ± 0.4^{a,b}	1.3 ± 0.1^{b}	1.2 ± 0.1^{b}
Total	**63.4 ± 5.2^{a}**	**53.4 ± 3.2^{a}**	**36.5 ± 0.7^{b}**	**40.4 ± 5.3^{a,b}**	**26.6 ± 3.8^{b}**	**30.6 ± 3.4^{b}**

Superscript letters indicate significant ($\alpha = 0.05$) depth differences within the control or elevated CO_2 treatment.

Table 4 Normalized aspen (*Populus tremuloides*) root amino acid-nitrogen (N) (± SE) amounts across treatments at the Rhinelander Free-Air CO_2 Enrichment (FACE) experiment within the different groups of amino acids for each root depth: 0–5 cm, 5–15 cm and 15–25 cm

	Normalized root amino acid-nitrogen (mg g^{-1} root N)					
	0–5 cm		5–15 cm		15–25 cm	
Amino acid groups	Control	Elevated CO_2	Control	Elevated CO_2	Control	Elevated CO_2
Nonpolar	**225.3 ± 4.6**	**238.5 ± 19.4**	**238.9 ± 21.6**	**210.9 ± 6.1**	**232.7 ± 15.6**	**239.4 ± 35.5**
Alanine	45.1 ± 0.6	47.0 ± 2.9	46.3 ± 3.7	42.5 ± 1.4	44.4 ± 2.5	49.0 ± 4.9
Glycine	49.3 ± 1.2	52.3 ± 4.4	50.6 ± 4.0	45.1 ± 1.2	46.2 ± 4.3	47.8 ± 8.0
Valine	30.4 ± 1.3	31.4 ± 2.2	32.3 ± 3.7	28.5 ± 0.5	31.2 ± 2.4	32.4 ± 5.3
Leucine	35.1 ± 0.8	38.9 ± 3.3	40.8 ± 4.0	34.3 ± 1.4	39.3 ± 2.7	39.3 ± 6.7
Isoleucine	18.1 ± 1.1	19.3 ± 1.7	19.7 ± 2.1	16.8 ± 0.4	19.9 ± 1.5	20.0 ± 3.0
Proline	30.5 ± 0.5	31.3 ± 3.1	32.0 ± 2.6	27.8 ± 0.7	33.8 ± 2.1	31.9 ± 5.3
Phenylalanine	16.9 ± 0.9	18.3 ± 1.9	17.2 ± 1.6	15.8 ± 0.6	17.9 ± 1.2	19.1 ± 2.9
Polar	**77.9 ± 3.9**	**81.6 ± 7.0**	**82.6 ± 4.9**	**74.2 ± 2.0**	**72.1 ± 9.2**	**84.0 ± 12.8**
Threonine	32.2 ± 1.7	34.7 ± 2.7	33.2 ± 1.3	31.5 ± 0.7	26.6 ± 3.7	34.8 ± 5.4
Serine	39.6 ± 1.5	42.2 ± 3.5	44.7 ± 3.5	39.0 ± 1.0	39.6 ± 6.4	44.6 ± 7.1
Tyrosine	6.1 ± 0.6	4.7 ± 0.8	4.8 ± 0.7	3.7 ± 0.3	5.9 ± 0.6	4.6 ± 0.4
Acidic	**106.4 ± 1.1**	**107.2 ± 11.2**	**110.0 ± 11.4**	**95.0 ± 3.4**	**87.2 ± 14.7**	**102.6 ± 17.4**
Aspartic acid	55.7 ± 0.9	56.1 ± 5.6	58.6 ± 6.5	50.2 ± 1.4	46.1 ± 8.3	55.4 ± 9.2
Glutamic acid	50.7 ± 0.3	51.0 ± 5.7	51.4 ± 4.9	44.9 ± 2.0	41.1 ± 6.3	47.3 ± 8.2
Basic	**30.5 ± 3.0**	**29.5 ± 3.1**	**30.0 ± 4.9**	**25.4 ± 0.4**	**29.1 ± 1.7**	**25.7 ± 4.6**
Lysine	30.5 ± 3.0	29.5 ± 3.1	30.0 ± 4.9	25.4 ± 0.4	29.1 ± 1.7	25.7 ± 4.6
Total	**440.1 ± 11.6**	**456.7 ± 40.5**	**461.6 ± 42.5**	**405.6 ± 11.5**	**421.0 ± 39.0**	**451.8 ± 70.1**

indicate different chemistry for deeper roots. Thicker roots in deeper soil of lower nutrient content and higher soil bulk density can penetrate soil more easily (Hutchings & John, 2004), and they probably have more structural compounds containing C than storage compounds in the form of proteins and amino acids.

Implications for SOM amino acid composition

As root and leaf litter are the initial primary contributors of C and N to SOM, and free amino acids comprise a large amount of the N leached from leaf litter (Chapin *et al.*, 1986), there would be a case for suggesting that changes in plant litter should

ultimately result in similar changes to SOM amino acids (Kuzyakov, 2001). However, Johnson & Pregitzer (2007) found that there was no significant effect of elevated CO_2 on the overall content of free amino acids in soil at the Rhinelander FACE site. They did, however, observe a minor but significant increase in aspartic acid and a decrease, although not significant, in valine. In our sampling, we found significant increases in both aspartic acid and valine in leaf litter amino acid C and amino acid N for just valine, and a decrease in root amino acid C valine. This might indicate that the valine observed previously in the free amino acids is coming from the root, whereas the aspartic acid found in the free amino acids is mainly from the leaf litter. Although Larson *et al.* (2002) observed no change in the protein degradation enzyme activity (leucine aminopeptidase) in soil at the Rhinelander FACE site, we showed that leaf amino acids decrease in proportional abundance and might not be the main contributor to water-soluble amino acids in soil, but rather that the main contributor might be root litter, in which we saw no changes in amino acids.

Recent analysis of the $\Delta^{14}C$–CO_2 respired from an incubation of Rhinelander FACE soil shows that the average age of C lost from the soil is *c.* 2 yr (Hopkins *et al.*, 2012) and that the most vulnerable pool of C to soil warming was years to decades old, meaning that most of the FACE input is respired and not stored. Based upon ^{14}C content of roots, Hopkins *et al.* (2012) speculated that recent root C comprised a majority of the C lost during the incubation and that microbial accessibility, governed by how long root organic matter remained as structurally intact tissue, was the main factor controlling the rate of release of much of the CO_2. Their assessment did not, however, have the resolution to distinguish CO_2 treatment effects in the dynamics of the decay of this fast-cycling, purportedly root-derived C. Additionally, Holmes *et al.* (2006) found that the new C inputs (roots and litter) from the elevated CO_2 at the Rhinelander FACE site were rapidly utilized by microbes, leading to increased N-mineralization rates (Holmes *et al.*, 2006), and Phillips *et al.* (2012) found evidence that root C turnover under elevated CO_2 at the Duke FACE site was enough to offset increased below-ground inputs as well as accelerated N cycling. Increased inputs as a result of root decomposition at lower depths under elevated CO_2 (Iversen, 2010) could also lead to priming of the C at greater soil depths and subsequently decrease the amount of C stored in the soils. Our results, which demonstrate changes in the proportion of amino acid C in leaves and a shift in the proportion of fine root amino acid C with depth, should be an influence on short-term soil C turnover if the decay dynamics evoked by Hopkins *et al.* (2012) are a governing factor. Ultimately, with a continued lower proportion of amino acid C in leaves and a lower proportional input of amino acid C to fine roots in the 0–5 cm depth, we would expect a slowing of the decay of the fast turnover pool and an accumulation of root and leaf particulate organic matter (POM) with higher C : N ratios. There were indications of this phenomenon in recent results from Hofmockel *et al.* (2011), who found that there was an increase in C in the POM fraction as a result of the Rhinelander FACE experiment,. However, even with lower proportional amino acid C, the increased NPP under elevated CO_2 would still be responsible for a net increase in amino acid protein to soil, as demonstrated by the following normalizations. By normalizing these amino acid concentrations to published leaf and fine root biomass C or N values (data from Zak *et al.*, 2011) for the year 2008, we can estimate the potential input of amino acid C or N into the soil. Our estimates suggest that the increased NPP resulting from elevated CO_2 can add 40 mg m^{-2} amino acid C from both leaf litter (27 mg m^{-2}) and root material (13 mg m^{-2}), along with an additional 32 mg m^{-2} of amino acid N from combined leaf litter (16 mg m^{-2}) and root material (16 mg m^{-2}) annually. This estimate assumes that all leaf litter is transmitted to soil and that our estimates of root-derived amino acids (extracted from combined living + dead) correspond to the 2008 biomass estimates. These estimates are likely conservative as our leaf litter tissue may have lost significant amino acid content since litter fall in 2008, and a significant proportion of the root isolates would contain dead and degraded tissue. These input estimates are also subject to interannual variability, as plant productivity, and perhaps amino acid content, changed during the early succession of the forest (King *et al.*, 2005).

Even with the potential net increase in amino acid C and N entering the soil, there is not necessarily a net increase in the uptake of amino acids by the trees under elevated CO_2. This was the conclusion of a study done at Duke FACE, which compared N uptake between elevated CO_2 and control plots using ^{15}N-NH_4^+ and ^{15}N-alanine (Hofmockel *et al.*, 2007). Although the potential plant amino acid N input to the soil does increase under elevated CO_2, it is apparently insufficient to supply the extra N needed to sustain the increased NPP under elevated CO_2. However, because of the greater root exudation under elevated CO_2 (Duke FACE; Phillips *et al.*, 2009), it could be expected that microbial decomposition of the increased root litter would also enhance and thus increase the availability of N to the trees (Phillips *et al.*, 2012). Understanding how changes in plant growth in response to elevated CO_2 impact the long-term dynamics of C and N in soil remains an important challenge. Plant litter is thought to be the main form of initial organic N input to the soil, and plant chemical composition can, in turn, influence trophic interactions, decomposition, and mixing rates, which ultimately feed back to affect the ecosystem and plant growth. Although the relative contribution of plant litter amino acid C and N is lower under elevated CO_2, the estimated annual input is also greater as a result of increased plant NPP. With the increased C and N, in the form amino acids, added to the soil each year under elevated CO_2, this input is potentially recycled by the trees by methods other than direct uptake in order to maintain the increased NPP at this FACE site.

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